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Lumen formation by epithelial cell lines in response to collagen overlay: A morphogenetic model in culture

(extracellular matrix/polarity/Madin–Darby canine kidney cell/normal murine mammary gland cell)

H. GLENN HALL, DEBORAH A. FARSON, AND MINA J. BISSELL

Laboratory of Cell Biology (83), Division of Biology and Medicine, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720

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ABSTRACT **Two cell lines—Madin–Darby canine kidney (MDCK) and normal murine mammary gland (NMuMG)—growing as monolayers on collagen gels were overlaid with another collagen gel. The cells responded to the overlay by undergoing reorganization resulting in the creation of lumina. MDCK cells formed lumina that coalesced to form large cavities comparable in size with a tubule. NMuMG cells formed clusters surrounding small lumina, which appeared similar to acini of glandular tissue. The characteristic arrangements, described here by light and electron microscopy, resembled the morphology of the tissues of cell line origin. MDCK cells, grown in the presence of serum, formed lumina whether or not serum was removed at the time of overlay, whereas NMuMG cells required either a nondialyzable component of serum or hormonal supplements in serum-free defined media. Lumen formation was delayed by MDCK cells in the presence of the glutamine analog 6-diazo-5-oxo-L-norleucine, but this compound did not affect NMuMG lumen formation. In both cell lines, lumen formation was unaffected by the absence of sulfate, the presence of an inhibitor of sulfated glycosaminoglycan synthesis, or an inhibitor of collagen synthesis. DNA synthesis accompanied lumen formation but was not required.**

The living stroma is typically separated from the exterior by a delimiting interface of epithelial cells. Epithelial cells can exist as stratified layers that may directly face the exterior, as for example in epidermis, or as a monolayer that may be folded into circular acini or ducts containing lumina continuous with the exterior. Certain specialized epithelial cells, such as those in the thyroid or cornea, border internal fluid-filled cavities that are closed to the exterior. The epithelial cell is functionally and morphologically polarized with respect to the two environments it separates. The basal cell surface is attached to extracellular matrix (ECM) material specialized as a layered basal lamina adjacent to the ECM of the stroma. In most epithelia, the opposite apical surface is free from an apposed extracellular layer. The rim of the apical surface is girdled by tight junctions to adjacent cells, so that the cell layer serves as a boundary to molecular diffusion. From the histological organization of epithelium, it appears that attachment to the ECM has a function in directing the polarity. This is strongly reinforced by the histology of invasive tumors. Metastatic epithelial tumor cells secrete hydrolytic enzymes that degrade the basal lamina before they invade the stroma (1). Tumor cells that have degraded the basal lamina lose their polarity, but if they maintain contact with undegraded basal lamina, they remain polar (2).

The purpose of the work described here is to establish morphogenetic models in culture in which ECM material, in this case type I collagen, induces epithelial rearrangement and thus provide a system for analysis of the biochemical steps involved in establishing epithelial polarity and organization. Monolayers of two epithelial cell lines were grown on a collagen gel and overlaid with another collagen gel. This creates an unusual situation for the cells, whereby the apical face comes into contact with a layer of ECM material. The cells respond to this challenge to their original polarity by creating new apical faces encircling newly formed lumina. This morphogenetic response is described and results are presented of inhibition experiments intended to identify necessary biochemical activities.

MATERIALS AND METHODS

Type I collagen gels were prepared by modification of an established procedure (3). Dissected rat tail tendons, after sterilization in 70% EtOH for 24 hr, were dissolved by stirring in 0.1% acetic acid for at least 48 hr (≈1 g of wet tendon per 150 ml) at 4°C. The collagen solution was cleared by centrifugation at 29,000 rpm (type 30 rotor, Beckman) for 1 hr. The supernate was mixed with 0.34 M NaOH and 5× concentrated minimal essential medium (8:1:2), spread on 35-mm plastic tissue culture dishes (1 ml per dish), placed in a CO_2 incubator, and allowed to gel.

Cell lines were obtained from Naval Biosciences Laboratory (University of California, Oakland, CA) (courtesy of Robert Owen). Madin–Darby canine kidney (MDCK) cells were maintained in minimal essential medium under 5% CO_2/95% air. Normal murine mammary gland (NMuMG) cells were maintained in Dulbecco's modified Eagle's medium under 10% CO_2/90% air. Unless otherwise stated, the medium was supplemented with 5% fetal calf serum. Cultures were transferred weekly to plastic dishes. For experiments, cells were seeded in 2 ml of medium at densities of 1.4×10^5/ml (MDCK) or 2.6×10^5/ml (NMuMG) on dishes containing collagen gels and allowed to grow for 4 days. After the medium was drawn off, the cell monolayer was rinsed with buffered saline solution and overlaid with the collagen mixture described above. Medium was added after the collagen had gelled. Lumen formation was monitored and photographed by phase-contrast microscopy; this report is based on observations of several hundred cultures.

For transmission electron microscopy, the cell layers contained in the collagen gels were fixed for 2 hr in 2% glutaraldehyde/0.1 M sodium cacodylate, pH 6.9, rinsed with cacodylate buffer, postfixed for 2 hr with 1% osmium tetroxide/0.1 M sodium cacodylate, and washed with water. The fixed collagen–cell layer was released from the dish, dehydrated by an increasing ethanol series and then in isopropanol, and embedded in a thin layer of Spurr's medium (Polysciences). Thin sections were stained with uranyl acetate/lead citrate.

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Abbreviations: ECM, extracellular matrix; MDCK, Madin–Darby canine kidney; NMuMG, normal murine mammary gland; GAG, glycosaminoglycan.

For measurement of DNA synthesis, [^{3}H]thymidine (5 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) was added for one 4-hr pulse on successive days in triplicate cultures. After labeling, the collagen–cell layer was homogenized in water (total vol, 5 ml). Duplicate aliquots (100 μl) of the homogenate were applied to GF/C glass fiber filters, dried, washed overnight with 5% trichloroacetic acid, rinsed with acetone, and assayed by liquid scintillation counting.

The inhibitors and analogs listed in Table 1 were obtained from Sigma. The hormone-supplemented serum-free medium formulas used were those of Taub *et al.* (4) for MDCK cells and of Salomon *et al.* (5) for NMuMG cells. Sulfate-free medium was basal medium (Eagle) diploid Earle's powder supplemented with 3% minimal essential medium 100× vitamin solution, 3% basal medium (Eagle) 100× amino acid solution (GIBCO), ferric nitrate (0.1 mg/ml), sodium pyruvate (110 mg/ml), glycine (30 mg/ml), and proline (42 mg/ml). The serum included in the sulfate-free medium was dialyzed for 2 days against the sulfate-free medium.

RESULTS

Morphological Studies. MDCK cells in culture are strongly polarized and form a tight epithelial monolayer that has been well characterized (6, 7). The polarized orientation of the cells with respect to a collagen gel substratum was challenged by providing an alternative collagen gel surface on the opposite (apical) side of the cells. After the cells had formed a monolayer on collagen (Fig. 1*A*), additional collagen solution was poured over the cells and allowed to gel. The upper gel often failed to attach to the surface of the MDCK cells and could be seen to float above the cell layer. To keep this gel immobilized and in close contact with the cells, the monolayer was overlaid before reaching confluency. The new (upper) collagen layer would adhere to the old (lower) one at the interspersed patches not covered by the cell monolayer. Within a few hours, the MDCK cell layer responded by contracting and withdrawing from the areas where the two gels came into contact. Cellular debris was left behind in the abandoned areas. After several hours, elevated darkened ridges and patches of cells, which appeared to be regions where the upper collagen gel had become immobilized by attachment to the cells, developed along the perimeter of the cell layer and throughout the culture. Within 12 hr after overlay, small refractile cavities appeared in these areas of apparent cell-to-gel attachment. Eventually, the areas of attachment spread to include the entire culture; the period of time varied from culture to culture. Within the next few days, numerous cavities formed over the entire MDCK cell layer, with smaller ones coalescing to form large ones (Fig. 1*B*). Under phase-contrast microscopy, the cavities appeared as open refractile "holes" in the culture. Only where the cavities were large was it apparent by phase-contrast microscopy that at least one cell layer was contained within the area of the cavity (Fig. 1*B*, a versus b). Sections perpendicular to embedded cell layers, examined by electron microscopy, showed that the cavities were, in fact, lumina, totally enclosed by new apical faces of two cell layers, one attached to the upper gel and another to the lower (Fig. 2). In MDCK cultures, lumina were surrounded by areas of close plasma membrane apposition resembling narrow tight junctions, and desmosomes were present along the lateral cell sides (Fig. 3). Cilia projected into the lumina (Fig. 4).

NMuMG cells (8) attached and spread readily on collagen gels. On both plastic and collagen substrata, NMuMG cells formed a heterogeneous-appearing monolayer consisting of large areas of spread cells interspersed with clusters and ridges of tightly packed cells (Fig. 1*C*). The overlaid collagen attached readily to the NMuMG cells, but the cell layer did not contract in response to the collagen. Within 12 hr after overlay, small lumina could be seen, predominantly in the dense cell patches that had existed in the monolayer before the overlay. Over the following week, lumina increased in number, enlarged and elongated, recruiting nearby cells into the arrangement. NMuMG lumina did not coalesce to the same extent as MDCK cells; their width was limited to a few cell diameters. Clusters of NMuMG lumina, with each surrounded by a single layer of cells, showed a striking resemblance to acini of glandular tissue (Fig. 1*D*). NMuMG lumina seen by electron microscopy (not

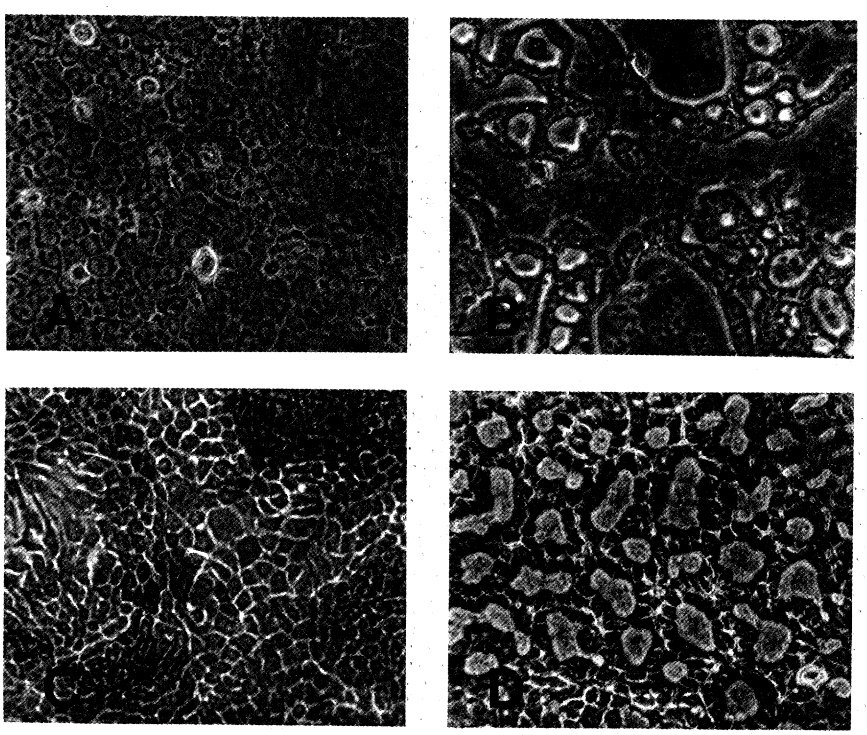

FIG. 1. Phase-contrast micrographs of cells grown in culture. (*A*) Monolayer of MDCK cells grown on a collagen gel. (*B*) MDCK cells were grown as in *A* and then overlaid with another collagen gel. Micrograph was taken 1 wk later. o, open spaces; a, small lumen; b, large lumen. (*C*) NMuMG cells grown on a collagen gel. (*D*) NMuMG cells were grown as in *C* and then overlaid with another collagen gel. Micrograph was taken 1 wk later.

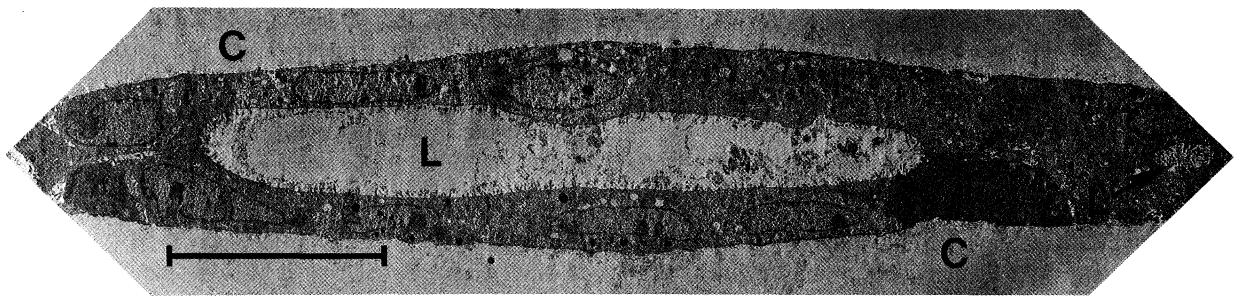

FIG. 2. Electron micrograph of a section perpendicular to a layer of MDCK cells grown on a collagen gel (C) and overlaid with collagen as in Fig. 1*B*. Note that the lumen (L) is completely surrounded by cells. Bar = 10 μm.

shown) appeared similar to, but smaller than, the MDCK lumen shown in Fig. 2.

MDCK cells grown on tissue culture plastic and overlaid with collagen maintained contact with the plastic dish and attached to the collagen gel over a period of 4–48 hr. The cell layer became greatly disorganized, with ridges and folds of cells. Only a few small lumina were formed. When monolayers of NMuMG cells were grown on plastic and overlaid with collagen, they immediately attached to the collagen and detached from the plastic dish. This released the collagen gel to float, so lumen formation could not be evaluated.

Effect of Serum Removal and Defined Media. When grown and maintained after overlay in hormone-supplemented serum-free medium, both cell lines formed lumina, but MDCK lumina were not as well developed as in the presence of serum. When serum was present during growth but removed at the time of collagen overlay, without hormone substitution, MDCK cells formed extensive lumina while NMuMG cells formed only a few small ones. The lumen inducibility of serum was found in the nondialyzable fraction; the dialyzable fraction was ineffective. Plasma fibronectin did not substitute for serum.

Inhibitor Studies. Two classes of inhibitors or analogs were used to identify macromolecules whose synthesis was required for lumen formation: (*i*) inhibitors of DNA, RNA, and protein synthesis and (*ii*) inhibitors of specific ECM components. DNA synthesis, as determined by the incorporation of [^{3}H]thymidine, accompanied lumen formation (Fig. 5). Thymidine incorporation continued at a high rate in NMuMG cells even after they reached confluency and after the collagen overlay. MDCK cells incorporated thymidine at a lower rate than NMuMG cells. The DNA synthesis inhibitor, 2′-azido-2′-deoxycytidine, did not prevent lumen formation in either cell line. In MDCK cultures, new cells apparently contribute to the lumen morphology because, in the presence of this inhibitor, there were more areas without cells and fewer areas with lumen arrangements, and the lumina were smaller. However, lumen morphology in NMuMG cells was not altered in the presence of this inhibitor, a puzzling finding in light of the high rate of DNA synthesis. Because a smaller percentage of cells contributed to lumina in comparison with the MDCK cultures, perhaps the cells not forming lumina incorporated most of the thymidine.

Actinomycin D and cycloheximide appeared to inhibit lumen formation by both cell types if added at the time of collagen overlay. These agents inhibited neither the initial attachment of MDCK cells to the collagen gel nor the contraction of the cell layer, but after a few hours in cycloheximide, the cells often released their attachment to the upper gel. Furthermore, given the time interval needed for lumen formation, these inhibitors may have limited life-sustaining functions before lumen formation could be seen. Thus, it was difficult to determine the effect of these agents on lumen formation *per se*.

Lumen formation was not affected in either cell line by the presence of the proline analog L-azetidine-2-carboxylic acid (9), indicating that collagen synthesis was not required. Lumen formation occurred in sulfate-free medium and in the presence of 4-methylumbelliferyl-β-D-xyloside (10), suggesting that sulfated glycosaminoglycan (GAG) synthesis is not needed. Ad-

FIG. 3. Electron micrograph of a section perpendicular to a layer of MDCK cells showing intercellular junctions adjacent to a small lumen (L). zo, Area resembling tight junction; za, zonula adherens; d, desmosome. Bar = 2 μm.

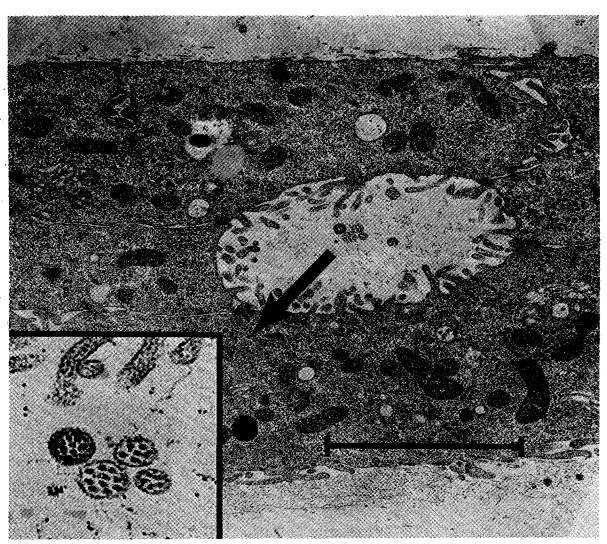

FIG. 4. Electron micrograph of a section perpendicular to a layer of MDCK cells showing a small lumen. (*Inset*) Cross sections of the distal ends of four cilia within the lumen. Bar = 4 μm.

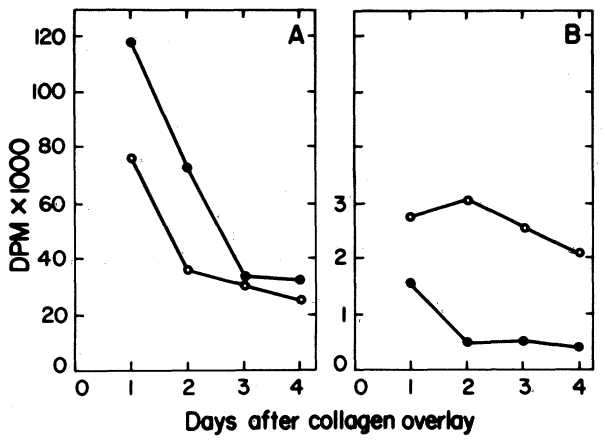

FIG. 5. Effect of collagen overlay on incorporation of [^{3}H]thymidine. Each point represents average incorporation in triplicate cultures during 4-hr periods. (*A*) NMuMG cells. (*B*) MDCK cells. ●, Cultures not overlaid; ○, cultures overlaid.

dition of the glutamine analog 6-diazo-5-oxo-L-norleucine (11), an inhibitor of amino sugar synthesis, at the time of initial cell seeding on the lower collagen substratum greatly affected cell morphology, particularly that of MDCK cells, which became very flattened and attenuated. On subsequent overlay with collagen, most of the MDCK cells kept their flattened appearance, but a few small scattered lumina were formed. No lumina were formed in NMuMG cultures treated the same way. Addition of the norleucine derivative at the time of overlay postponed lumen formation in MDCK cultures by several days but did not inhibit lumen formation in NMuMG cultures. The results of the inhibitor studies are summarized in Table 1.

DISCUSSION

Collagen and other ECM components have been shown to exert a profound influence on the morphology and growth of cells. Recent reviews discuss the role of collagen in development (12), the effects of collagen matrices on cell adhesion and growth (13),

Table 1. Effect of analogs, inhibitors, and media modifications on lumen formation

Addition or modification	MDCK cells	NMuMG cells
2′-Azido-2′-deoxycytidine (50 μg/ml)	Size reduced	No effect
Actinomycin D (0.5 μg/ml)	Inhibition*	Inhibition*
Cycloheximide (10 μg/ml)	Inhibition*	Inhibition*
L-Azetidine-2-carboxylic acid (25 μg/ml)	No effect	No effect
4-Methylumbelliferyl-β-D-xyloside (0.1 mM)	No effect	No effect
6-Diazo-5-oxo-L-norleucine (5 μg/ml)	Formation delayed	No effect
Sulfate-free medium	No effect	No effect
Serum removal	No effect†	Inhibition
Fibronectin (5 μg/ml) in absence of serum	Not applicable	Inhibition not overcome
Serum-free defined hormone-supplemented medium	Not applicable†	Inhibition overcome

Additions and modifications were made at the time of collagen overlay.
* Possibility of cell death or damage.
† If serum was replaced by hormones at the time of initial cell seeding, lumina were not as well developed.

and the effect of ECM on gene expression (14). Of particular relevance here are culture studies in which collagen was shown to affect cell organization and morphology. Primary mammary epithelial cells grown on collagen gels underwent shape and functional changes after the gels were released from the culture dish (3). Growth of primary mammary epithelial cells was sustained by embedding the cells in a collagen gel in which they formed three-dimensional tissue-like structures with branching tubules (15, 16).

In the overlay procedure used in this study, the cell layer is essentially embedded in collagen. However, in contrast to the studies described above, in which the structures became established as the cells grew, here the apical face of an established epithelium was brought into contact with the collagen. To resolve the apparent conflict created, the epithelium rearranged to a tissue-like structure compatible with being surrounded by a matrix. The distinct lumen morphologies formed by the two cell lines, the broad lumina of the MDCK cells and the rounded acini and short ducts of NMuMG cells, resembled the morphologies of the tissues of cell line origin: MDCK derived from distal kidney tubule and NMuMG derived from mammary gland. The different responses may be an inherent property of the cell types retained after many passages in culture. MDCK cells have previously been shown to have retained the ability to form lumina on injection into baby *nude* mice (17). During the course of the work reported here, results were reported of collagen-overlay experiments with *primary* thyroid epithelial cells (18). The thyroid cells responded in a similar way by forming follicles. Recent studies have further explored the morphological capability of primary mammary epithelial cells embedded in collagen (19). The combination of a cloned myoepithelial cell line and a cloned epithelial line, but neither line alone, resulted in tubule formation that penetrated the collagen gel. Primary epithelial cells might be expected to retain some morphogenetic capabilities in culture whereas established cell lines would be expected to lose them. Indeed, in our preliminary work, HBL-100 cells, a human mammary cell line classified as epithelial, did not respond to collagen overlay by forming lumina. Thus, morphogenetic qualities may be lost in some cell lines whereas in other lines, as shown here, not only can they be retained after numerous passages, they can be expressed in a tissue-specific manner.

Although special chemical and mechanical properties of ECM components probably contribute to epithelial formation, a stable surface to which the cells can attach may be all that is necessary to direct polarity. Simply by attaching to a flat surface, a cell can become polarized. For cells in culture, contact sites with the plastic culture dish exist on one side only, and in fibroblasts, cytoskeletal elements are localized at these regions (20). Since ECM material of the stroma surrounds fibroblasts *in vivo*, the culture-dish-induced polarity may be atypical for this cell type. On the other hand, the polarity of epithelial cells with respect to the plastic substratum corresponds to the normal *in vivo* polarized association with the basal lamina, which can be viewed as a substratum.

MDCK cells grown on plastic and overlaid with collagen form, at best, only a few small lumina. However, thyroid cells do form follicles if sandwiched between collagen and glass (18), suggesting that both materials function adequately as an adhesive surface in directing polarity. In some cases, formation of an epithelium appears to require no substratum other than adjacent cell surfaces. Dissociated cells of the starfish blastula can organize into an epithelium without the aid of an extracellular layer (21). When MDCK cells are initially plated onto collagen, small transient lumina form in cell clusters before the cells spread out over the collagen gel. Primary thyroid cells sus-

pended in low-serum medium organize into cyst-like (apical end out) structures without a supporting extracellular layer (22). However, the thyroid cysts reverse polarity to form follicles when embedded in a collagen gel (18). The formation of polarized epithelium may thus be an intrinsic cellular property that can be expressed in small rounded clusters of cells. This property may be dependent solely on cell–cell interactions but capable of modification by an extracellular layer when it is present. Alternatively, the presence of extracellular material on cell surfaces may be required to direct polarity, but small amounts of the material may be adequate and need not exist as a visible layer. For example, the polarity reversal of thyroid cysts also occurs in the presence of high concentrations of serum (22). Soluble ECM components, including collagen, have been shown to have a profound effect on epithelial surface morphology (23). In culture, cells may establish their polarity in response to small amounts of self-synthesized ECM material adsorbed to the artificial surface. The effect of the adsorbed material would then be difficult to separate from the effect of a simple surface. Although, minor amounts of ECM material may direct polarity and lumen formation in small clusters of cells to create a minimal epithelium, a layer of ECM material providing a flexible and permeable surface may be necessary to extend and modify this basic epithelium to form large lumina and tissues.

During the process of lumen formation, cell attachment to type I collagen may be direct (24, 25) or may be mediated by ECM components such as fibronectin, type IV collagen, laminin, and GAGs (for review, see ref. 13). Plasma fibronectin appears not to be involved in the cell–collagen association that induces lumen formation, nor is there a need for newly synthesized collagen. GAG synthesis by NMuMG cells has been reported (26), and we have found sulfated GAG synthesis by both MDCK and NMuMG cells (unpublished results). Our results suggest that synthesis of sulfated GAGs is not required for lumen formation, although a possible role for the nonsulfated hyaluronic acid has not been ruled out. It is important to recognize that a number of such components may be necessary for lumen formation and that they may be present in the crude collagen gel preparation. In fact, our preliminary results indicate that purified collagen (Vitrogen) is not as effective as rat tail collagen in allowing lumen formation.

NMuMG cells attach more readily to the collagen overlay than do MDCK cells. What determines this difference is not yet known, but it may represent cell surface topographical differences in the distribution of collagen-binding components. In the highly organized MDCK monolayer, only the apical surface, which may not be able to bind collagen, comes into initial contact with the overlay, whereas in the more disorganized monolayer of NMuMG cells, cell surface domains may not be as segregated and collagen-binding components may be present at the upper surface.

We have demonstrated that available easily maintained well-characterized epithelial cell lines still retain morphogenetic capabilities that can be analyzed as a model system in culture. This relatively simple model may prove valuable in studying the steps involved in cellular organization into tissues. The response is inducible and proceeds over a period of time suitable for experimental dissection of the steps involved. The possible rearrangements of cell surface components can be studied without need of prior enzymatic dissociation of the cells. The highly developed epithelium of MDCK cells, with well-defined membrane domains, will be particularly valuable in studying possible topographical rearrangements of surface components, membrane recycling, vectorial transport, cytoskeletal rearrangements, and the dynamics of junction formation and distribution. Comparison of cell lines exhibiting different morphological responses may reveal differences in biochemical activities that are responsible. Mechanisms that may be required for lumen formation in general may be distinguished from those generating specific morphologies, as exemplified by the two cell lines used in this study.

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